



EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2014. Scientific Opinion on xylanase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FB)

EFSA Journal

Link to article, DOI:
[10.2903/j.efsa.2014.3645](https://doi.org/10.2903/j.efsa.2014.3645)

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
EFSA Journal (2014). *EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2014. Scientific Opinion on xylanase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FB)*. European Food Safety Authority. EFSA Journal Vol. 12(5) No. 3645
<https://doi.org/10.2903/j.efsa.2014.3645>

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SCIENTIFIC OPINION

Scientific Opinion on Xylanase from a Genetically Modified Strain of *Aspergillus oryzae* (strain NZYM-FB)^{1,2}

EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids (CEF)^{3,4}

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The food enzyme considered in this opinion is a xylanase (endo-1,4- β -xylanase; EC 3.2.1.8) produced with a genetically modified strain of *Aspergillus oryzae*. The genetic modifications do not raise safety concern. The food enzyme contains neither the production organism nor recombinant DNA. The xylanase is intended to be used in a number of food manufacturing processes, such as starch processing, beverage alcohol (distilling), brewing, baking and other cereal based processes. The dietary exposure was assessed according to the Budget method. The food enzyme did not induce gene mutations in bacteria nor chromosome aberrations in human peripheral blood lymphocytes. Therefore, there is no concern with respect to genotoxicity. The systemic toxicity was assessed by means of a 90-day subchronic oral toxicity study in rodents. A No Observed Adverse Effect Level was derived, which compared with the dietary exposure results in a sufficiently high Margin of Exposure. The allergenicity was evaluated by searching for similarity of the amino acid sequence to those of known allergens. The Panel considered that the likelihood of food allergic reactions to the enzyme is low and therefore does not raise safety concern. Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided and the toxicological studies, this food enzyme does not raise safety concern under the intended conditions of use.

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¹ On request from European Commission, Question No EFSA-Q-2012-00897, adopted on 09 April 2014.

² This scientific opinion replaces the previously published summary of the opinion following the provisions of article 12(3) of Regulation (EC) No 1331/2008.

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Suggested citation: EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2014. Scientific Opinion on xylanase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FB). EFSA Journal 2014;12(5):3645, 17 pp. doi:10.2903/j.efsa.2014.3645

Available online: www.efsa.europa.eu/efsajournal

KEY WORDS

food enzyme, xylanase, endo-1,4- β -xylanase, EC 3.2.1.8, 1,4- β -D-Xylan xylanohydrolase, *Aspergillus oryzae*, genetically modified microorganism

SUMMARY

Following a request from the European Commission, the EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the food enzyme xylanase (endo-1,4- β -xylanase; EC 3.2.1.8) produced with the genetically modified *Aspergillus oryzae* strain NZYM-FB.

The *A. oryzae* parental strain has a long history of use for the production of food enzymes. It has been modified in order to produce and secrete xylanase and to prevent or decrease the production of undesirable secondary metabolites. The genetic modifications do not raise safety concern.

The food enzyme contains neither the production organism nor recombinant DNA, given the limits of detection.

The food enzyme has been characterised by determining the temperature and pH optima and the thermo-stability. Its composition is verified by measuring the content of protein, ash, water, heavy metals and Total Organic Solids. The *A. oryzae* parental strain has been modified in order to prevent the production of cyclopiazonic acid and to decrease the potential production of kojic acid. The absence of cyclopiazonic acid, β -nitropropionic acid and kojic acid was demonstrated, given the limits of detection.

The food enzyme is intended to be used in a number of food manufacturing processes, such as starch processing, beverage alcohol (distilling), brewing, baking and other cereal based processes. The typical uses and the use levels recommended for specific food processes have been provided.

The Theoretical Maximum Daily Intake was calculated according to the Budget method.

The genotoxicity of the food enzyme was assessed by means of two *in vitro* assays (gene mutations in bacteria and chromosome aberrations in human lymphocytes). The food enzyme, produced with the genetically modified *A. oryzae* strain NZYM-FB, did not induce gene mutations in bacteria with or without metabolic activation when tested under the conditions employed in the study as presented by the applicant. Neither did it induce chromosome aberrations in cultured human blood lymphocytes under the test conditions employed for this study. The systemic toxicity was assessed by means of a 90-day subchronic oral toxicity study in rodents. A No Observed Adverse Effect Level (NOAEL) was derived, which compared with the dietary exposure results in a sufficiently high Margin of Exposure.

The CEF Panel considers that the likelihood of food allergic reactions to this xylanase produced with this genetically modified strain of *A. oryzae* is low and therefore does not raise safety concern.

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided and the toxicological studies, the Panel concluded that this food enzyme does not raise safety concern under the intended conditions of use.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Only food enzymes included in the Union list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008⁵ on food enzymes. According to Regulation (EC) No 1332/2008 on food enzymes, a food enzyme which falls within the scope of Regulation (EC) No 1829/2003⁶ on genetically modified food and feed should be authorised in accordance with that Regulation as well as under this Regulation.

An application has been introduced by the company Novozymes A/S for the authorisation of the food enzyme xylanase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FB).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011⁷ implementing Regulation (EC) No 1331/2008⁸, the Commission has verified that the application falls within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority to carry out the safety assessment on the food enzyme xylanase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FB) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

⁵ Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/199, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, p. 7-15.

⁶ Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003 p. 1-23.

⁷ Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.03.2011, p. 15-24.

⁸ Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p. 1-6.

ASSESSMENT

1. Introduction

Before January 2009 food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009 the Regulation (EC) No 1332/2008 on food enzymes entered into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. The Regulation (EC) No 1331/2008 established Union procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall only be authorised if it is demonstrated that:

- (i) it does not pose a safety concern to the health of the consumer at the level of use proposed;
- (ii) there is a reasonable technological need, and
- (iii) its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on that market as well as all new food enzymes shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and an approval via a Union list.

The Guidance on submission of a dossier on a food enzyme for evaluation by EFSA (EFSA, 2009) lays down the administrative, technical and toxicological data required.

In the case of enzymes produced with genetically modified microorganisms (GMM) the guidance on the risk assessment of GMM and their products intended for food and feed use (EFSA GMO Panel, 2011) applies.

The assessment of a GMM aims to evaluate the safety of the food enzyme related to the genetic modifications. This starts from the evaluation of the safety of the parental/recipient and donor strains, e.g. regarding their capability to produce undesirable metabolites. It also includes the assessment of the genetic modifications, specifically of the introduction of genes of concern and the genetic stability. Finally, the presence of the production organism and its recombinant DNA in the final product is assessed. Special attention is paid to the presence of any recombinant genes of concern (e.g. antibiotic resistance genes), introduced during the development of the production strain. If the absence of the production strain and the recombinant DNA has been confirmed, no extensive environmental risk assessment of the enzyme product is required.

The following evaluation applies to the xylanase (endo-1,4- β -xylanase; EC 3.2.1.8) produced with the genetically modified *A. oryzae* strain NZYM-FB.

2. Information on existing authorisations and evaluations

The applicant reports that the Danish, French and South Korean authorities have evaluated and authorised the use of the xylanase food enzyme produced with the genetically modified *A. oryzae* strain NZYM-FB in a number of food manufacturing processes, such as starch processing, alcohol distilling, brewing and baking processes. Only the Danish authority specified the conditions of use, i.e. max. 200 FXU(S)⁹/kg wheat flour for starch and gluten products and max. 100 FXU(S)/kg for barley or malt.

⁹ Fungal Xylanase Units

3. Technical data

3.1. Identity of the food enzyme

IUBMB nomenclature:	Endo-1,4- β -xylanase
Systematic name:	1,4- β -D-Xylan xylanohydrolase
Synonyms:	Xylanase; endo-1,4-D- β -xylanase
IUBMB No:	EC 3.2.1.8
CAS No:	9025-57-4
EINECS No:	232-800-2.

3.2. Chemical parameters

The xylanase produced with the genetically modified *A. oryzae* strain NZYM-FB is a single polypeptide chain of 384 amino acids. The molecular mass derived from the amino acid sequence was calculated to be 40.8 kDa.

Data on the chemical parameters and the protein homogeneity status of the food enzyme have been provided for three enzyme preparation batches and for the food enzyme batch (PPJ 6867) used for the toxicological tests (Table 1).

The average Total Organic Solids (TOS) of the three enzyme preparation batches was 10.6 % (w/w); the values ranged from 9.3 to 11.6 % (Table 1). TOS is a calculated value derived from 100 % minus % water % minus % ash and minus % diluents (stabilisation and formulation ingredients). The three enzyme preparation batches presented in Table 1 are ultra-filtered concentrates, which were stabilised and preserved by addition of potassium sorbate, sodium benzoate, sorbitol and glycerol. The batch used for toxicological tests does not contain any diluents.

The average specific activity of the enzyme preparations expressed as ratio enzyme activity/mg TOS was 23.6 FXU(S)/mg TOS; the values ranged from 21.5 to 26.1 FXU(S)/mg TOS (Table 1). Considering the low variability of the activities as well as the specific activities in the three enzyme preparations, the average activity/mg TOS value of 23.6 FXU(S)/mg TOS was used for subsequent calculations.

A. oryzae, as a species, is known to have the potential to produce undesirable secondary metabolites such as cyclopiazonic acid (CPA), β -nitropropionic acid (NPA) and kojic acid (KA) (Blumenthal, 2004). Accordingly, these mycotoxins must be checked in the final food enzyme from *A. oryzae* (FAO/WHO, 2006). *A. oryzae* as a species belongs to the *Aspergillus flavus* group which is known to have a gene cascade for the biosynthesis of aflatoxins, but under any known fermentation conditions, *A. oryzae* strains never produced aflatoxins (Blumenthal, 2004; Lee et al., 1991). The parental strain has been modified by γ -irradiation mutagenesis, resulting in the deletion of gene clusters required for the synthesis of the mycotoxins cyclopiazonic acid (*cpa*) and aflatoxin (*afl*). UV irradiation mutagenesis resulted in a reduced potential (15 % of the original) to produce kojic acid. None of these three species-specific mycotoxins (CPA, NPA and KA) were detected in the food enzyme (Table 1) by mass spectrometry (LC-MS/MS).

The protein homogeneity status of the food enzyme was also investigated by SDS-PAGE analysis. The apparent molecular mass based on this technique is about 45 kDa (compared to the calculated molecular mass of 40.8 kDa). According to the applicant, this is most likely due to glycosylation, a common feature of proteins of *Aspergillus*. The gels presented for the four batches were comparable;

variations in the relative amounts of the protein bands were suggested by the applicant to be caused by different glycosylation patterns.

The food enzyme was tested for other enzyme activities, i.e. alpha-amylase, glucoamylase, protease and lipase activities, which were below the detection limits of the employed methods (Table 1).

Table 1: Compositional data of the food enzyme

Parameter	Unit	Batches			
		CDG00157	CDG00158	CDG00159	PPJ 6867 ^(a)
Xylanase activity	FXU(S)/g batch ^(b)	2 500	2 490	2 430	2 000
Protein	% (w/w)	7.1	6.9	7.3	7.1
Ash	% (w/w)	0.3	< 0.3	0.3	2.3
Water	% (w/w)	61.7	61.6	61.8	87.6
Total Organic Solids (TOS) ^(c)	% (w/w)	10.8	11.6	9.3	10.1
Activity/mg TOS	FXU(S)/mg TOS	23.1	21.5	26.1	19.8
Pb	mg/kg batch	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
As	mg/kg batch	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Cd	mg/kg batch	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Hg	mg/kg batch	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Antimicrobial activity		ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Production strain	CFU per g batch	NA ^(e)	NA ^(e)	NA ^(e)	ND ^(d)
Cyclopiazonic acid	mg/kg batch	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Kojic acid	mg/kg batch	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
β-nitropropionic acid	mg/kg batch	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Antifoam agents	mg/kg batch	352	1 307	781	1 410
Alpha-amylase	FAU(F)/g batch ^(f)	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Glucoamylase	AGU/g batch ^(g)	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Protease	HUT/g batch ^(h)	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)
Lipase	KLU/g batch ⁽ⁱ⁾	ND ^(d)	ND ^(d)	ND ^(d)	ND ^(d)

(a): Batch used for the toxicological tests (Food enzyme before stabilisation and formulation (See footnote c)).

(b): FXU(S): Fungal Xylanase Units (relative to an internal enzyme standard “S”) (see Section 3.3).

(c): TOS calculated as 100 % - % water - % ash - % diluents (Diluents: potassium sorbate, sodium benzoate, sorbitol and glycerol used for stabilisation of the enzyme preparation batches CDG00157, CDG00158 and CDG00159 in accordance with Commission Regulation (EU) No 1130/2011¹⁰).

(d): ND: Not detected: below the limits of detection (Pb: 0.5 mg/kg, for the food enzyme batch PPJ 6867 the limit of detection was 1 mg/kg; As: 0.1 mg/kg; Cd: 0.05 mg/kg; Hg: 0.03 mg/kg; cyclopiazonic acid: 0.01 mg/kg; kojic acid: 0.2 mg/kg, for the batch PPJ 6867 the limit of detection was 2 mg/kg; β-nitropropionic acid: 0.3 mg/kg; alpha-amylase: 0.34 FAU(F)/g; glucoamylase: 0.825 AGU/g; protease: 196 HUT/g; lipase: 0.02 KLU/g; antimicrobial activity: inhibition zone diameters ≤ 16 mm imply that the antimicrobial activity was absent (FAO/WHO, 2006)). According to the applicant, the differences in the detection limits for lead and kojic acid in the three enzyme preparations and in the food enzyme batch used for toxicological testing are due to differences in the performances of the methods at the respective point in time of the analysis.

(e): NA: Not analysed.

(f): FAU(F): Fungal alpha-Amylase Units (relative to an internal enzyme standard “F”).

(g): AGU: AmyloGlucosidase Units.

(h): HUT: Hemoglobin Units Tyrosine.

(i): KLU: Kilo Lipase Units.

¹⁰ Commission Regulation (EU) No 1130/2011 of 11 November 2011 amending Annex III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives by establishing a Union list of food additives approved for use in food additives, food enzymes, food flavourings and nutrients. OJ L 295, 12.11.2011, p. 178-204.

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample, and total coliforms are not more than 30 CFU (Colony Forming Units) per gram.

The applicant has provided information on the identity of the antifoam agents and the Panel considers the use of these as of no safety concern.

The provided data regarding compositional batch-to-batch-variability are considered sufficient. Table 1 shows that the food enzyme batch PPJ 6867 used for the toxicological assays has a lower activity/mg TOS and a higher level of inorganic constituents and antifoam agents. Consequently, this food enzyme batch PPJ 6867 is cruder than the three enzyme preparations and its use for the toxicological testing is considered suitable.

3.3. Properties of the food enzyme

Xylanases catalyse the hydrolysis of 1,4- β -D-xylosidic endo-linkages in xylan (including arabinoxylan, which is xylan branched with arabinose) resulting in the generation of (1 \rightarrow 4)- β -D-xylan oligosaccharides of different lengths. The xylanase of *A. oryzae* strain NZYM-FB does not require co-factors.

The enzymatic activity is quantified based on the hydrolysis of wheat arabinoxylan and is expressed in Fungal Xylanase Units/g (FXU(S)/g). The xylanase activity is measured relative to an internal enzyme standard ("S"). The analytical principle is based on hydrolysis of the arabinoxylan to reducing carbohydrates (reaction conditions: pH = 6.0, T = 50 °C, incubation time = 5 minutes). The reaction is stopped by adding *p*-hydroxy benzoic acid hydrazide (PAHBAH) and bismuth (III)-tartrate which complexes with the reducing carbohydrates, producing a colour that is detected spectrophotometrically at 405 nm.

The food enzyme has been characterised regarding its activity depending on temperature and pH. The xylanase is active at temperatures up to 80 °C (with an optimum of 60 - 70 °C at pH 6.0) and within a pH range of 3 to 8 (with an optimum of pH 3 - 4 at 37 °C). The thermo-stability of the food enzyme was tested over the range of 25 °C to 90 °C after a pre-incubation at the different temperatures at pH 6.0 for 30 minutes. The activity itself was measured under standard conditions. The food enzyme shows 50 % residual activity at 60 - 65 °C after 30 minutes incubation and above 80 °C, no enzyme activity remains after 30 minutes.

3.4. Information on the source materials

3.4.1. Information relating to the genetically modified microorganism

3.4.1.1. Characteristics of the recipient and parental microorganisms

The production organism is the fungus *A. oryzae*. *A. oryzae* strains are not qualified as QPS (Qualified Presumption of Safety) because of the potential of mycotoxin production (EFSA BIOHAZ, 2012). The parental strain, *A. oryzae* A1560, has a long history of use for the production of food enzymes. The recipient strain *A. oryzae* BECh2, has been developed from the parental strain, *A. oryzae* A1560, through a series of modification steps including classical mutagenesis and genetic modification (the latter not described for confidentiality reasons).

The classical mutagenesis steps included γ -irradiation mutagenesis, resulting in the deletion of gene clusters required for the synthesis of the mycotoxins cyclopiazonic acid (*cpa*) and aflatoxin (*afl*), and UV irradiation mutagenesis, resulting in a drastically reduced potential (15 % of the original) to produce kojic acid. A Southern blot analysis confirmed the absence of antibiotic resistance genes which were used during the genetic modification of the recipient strain.

3.4.1.2. Characteristics of the donor organisms

The plasmid vector used for the transformation of the recipient strain contained elements to control the expression of the gene of interest as well as ensure integration of the introduced DNA into the recipient strain chromosome. The DNA introduced into the recipient strain did not contain antibiotic resistance genes.

3.4.1.3. Description of the genetic modification process

The production strain *A. oryzae* NZYM-FB was developed from the recipient strain BECh2 through transformation with a plasmid vector and selection of the transformants on appropriate medium.

The production strain differs from the recipient strain by the synthesis and secretion of endo-1,4- β -xylanase.

3.4.1.4. Safety aspects of the genetic modification: Information relating to the GMM and comparison of the GMM with its conventional counterpart

In comparison to the parental strain A1560, the recipient strain has lost the potential to produce cyclopiazonic acid and has a reduced potential to produce kojic acid.

The final production strain NZYM-FB differs from the recipient strain by the synthesis and secretion of endo-1,4- β -xylanase.

The presence of the gene encoding endo-1,4- β -xylanase in the NZYM-FB strain was verified by Southern analysis; this indicated that multiple copies of the full-length gene were integrated in the chromosomal DNA of the recipient. In order to estimate the number of copies, a dot blot analysis was carried out.

Southern analysis of the DNA production strain from three independent enzyme preparation batches at the end of pilot scale fermentation confirmed the genetic stability of the genetic modifications.

The genetic modifications do not raise safety concern.

3.4.1.5. Safety for the environment

Neither the production strain nor its recombinant DNA were detected in the final product (see Section 3.5.2). Accordingly, as the food enzyme belongs to Category 2 of the guidance on risk assessment of genetically modified microorganisms and their products (EFSA GMO Panel, 2011), environmental exposure to the genetically modified microorganism or its DNA is negligible and hence no further environmental risk assessment is required.

3.5. Manufacturing process

The manufacturing process includes a fermentation process and downstream processing. A comprehensive dataset related to the manufacturing process including a flow diagram was provided. The food enzyme is manufactured according to the Regulation (EC) No 852/2004¹¹. According to the applicant the manufacturing process is certified according to Food Safety Systems Certification 22000 (FSSC 22000) and ISO 9001.

3.5.1. Information relating to the fermentation process

The food enzyme is produced by a pure culture in contained submerged fed-batch fermentation with conventional process controls in place. The identity and the purity of the culture are checked at each transfer step from frozen vials to the end of fermentation.

¹¹ Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, p. 3-21.

3.5.2. Information relating to the downstream processing

The downstream processing includes recovery, purification and concentration. The food enzyme produced is recovered from the fermentation broth after biomass separation via filtration. Further purification and concentration involve a series of filtration steps, including ultrafiltration and final germ filtration.

The production strain could not be detected in a test volume of 1 g of three independent liquid batches tested in triplicate by liquid culturing in non-selective medium for 4 days (for resuscitation) followed by growth on selective solid agar plates for 4 days at a suitable temperature favouring the growth of the production strain. No recombinant DNA was detected starting with 1 g of three samples of the concentrated product before formulation obtained from three independent production batches and tested in triplicate. Analysis was performed by PCR, amplifying a 1 076 bp recombinant fragment spanning the deletion of an endogenous gene specific for the strain lineage, introduced in the first genetic modification step of the parental strain.

3.6. Reaction and fate in food

The xylanase catalyses the hydrolysis of 1,4- β -D-xylosidic endo-linkages in xylan (including arabinoxylan, which is xylan branched with arabinose) resulting in the production of (1 \rightarrow 4)- β -D-xylan oligosaccharides of different lengths and xylose.

Xylanase is specific in its action, not known to catalyse other reactions than this endo-hydrolysis of xylans to shorter xylans chains, xylo-oligosaccharides and xylose. These reaction products are naturally present in xylan-containing foods. The data and information provided indicate that the xylanase is denatured and/or removed during processing under the intended use conditions. The food enzyme was tested for other enzyme activities, i.e. alpha-amylase, glucoamylase, protease and lipase activities, which were below the detection limits of the employed methods. Therefore, no unintended products resulting from the xylanase and any side activities are to be expected.

3.7. Case of need and intended conditions of use

The food enzyme is intended to be used in a number of food manufacturing processes. Typical uses provided by the applicant are listed in Table 2.

Table 2: Typical uses and recommended maximum use levels of the food enzyme as provided by the applicant

Process	Recommended dosage of the food enzyme
Starch processing	Up to 600 FXU(S)/kg of wheat flour or cereal dry matter
Beverage alcohol (distilling) processes	Up to 600 FXU(S)/kg of wheat flour or cereal dry matter
Brewing processes and other cereal-based beverage processes	Up to 400 FXU(S)/kg of grits (e.g. barley, malt, wheat, rice), corresponding to 16.9 mg TOS/kg (400 FXU(S)/23.6 FXU(S)/mg TOS (23.6 is the average of the three enzyme preparation batches))
Baking processes and other cereal-based processes	Up to 650 FXU(S)/kg of wheat flour or cereal dry matter corresponding to 27.5 mg TOS/kg (650 FXU(S)/23.6 FXU(S)/mg TOS (23.6 is the average of the three enzyme preparation batches))

Wheat, barley and other cereals are highly complex raw materials causing technical difficulties during processing.

In starch processing, the xylanase is added to the water before mixing the raw materials in both the wheat dough process and the wheat decanter process. It is used in order to improve the processes for the separation of starch, gluten and fibres, mainly by removing the arabinoxylans and reducing the viscosity of the water soluble arabinoxylans, resulting in energy savings due to less use of process water and lower evaporation costs.

In the beverage alcohol (distilling) process, the xylanase is intended to be used in order to improve the manufacturing steps by reducing the viscosity, thereby improving heat exchange and centrifugal separation and to increase the yield of fermentable sugars from xylan hydrolysis. The xylanase is mainly added during the slurry mixing. In addition, the xylanase can also be added at the liquefaction or the fermentation steps.

In the brewing process and other cereal based beverage processes, the xylanase is mainly used in order to ease filtrations and increase the flexibility in the choice of raw materials. The xylanase is added during the mashing step or alternatively during the cooking/liquefaction step and to get more uniform and predictable processes.

In baking and other cereal based processes, the xylanase is added to raw materials during the preparation of the dough. It is used to hydrolyse arabinoxylans which react with gluten and water and thus contribute to the viscosity of the dough. Its use gives improved crumb structure and facilitates the handling of the dough, resulting in more uniform products. In other cereal based processes, the benefit of the use of xylanase is also a faster drying step.

According to the applicant, the food enzyme is used at the minimum dosage necessary to achieve the desired reaction according to Good Manufacturing Practice (GMP). The dosage applied in practice by a food manufacturer depends on the particular process (see Table 2).

4. Dietary exposure

The exposure assessment was performed according to the Budget method (FAO/WHO, 2009). This method has been used as a screening method in assessing food additives by the JECFA (FAO/WHO, 2001) and for assessments within the EU Scientific Cooperation (SCOOP) Task 4.2 (EC, 1998). The approach is based on the maximising assumptions that 25 % of consumed solid foods and 25 % of consumed beverages would be manufactured using the food enzyme and that all of the food enzyme would remain in the final processed food products. It is expected that the enzyme would be inactivated and/or residues of the food enzyme would be removed in the course of the production and processing of final food products. However, considering that the xylan hydrolysates resulting from the use of the food enzyme may be used in a variety of applications as ingredients of food and/or beverages, the Panel considered it appropriate to use this conservative upper-bound screening approach as part of a tiered approach to estimate the dietary exposure.

The Panel followed the principles of the stepwise approach, which was used to assess Theoretical Maximum Daily Intake (TMDI).

The estimation is based on the following assumptions:

- The level of consumption of foods and of non-milk beverages:
The levels of consumption of foods and beverages considered are maximum physiological levels that can be consumed each day - i.e. the daily consumption of 0.1 L/kg body weight (bw) of non-milk beverages and the daily consumption of 100 kcal/kg bw from foods (equivalent to 0.05 kg/kg bw based on an estimated energy density of 2 kcal/g). These levels correspond to the daily consumption of 6 L of non-milk beverages and 3 kg of food by a person with a body weight of 60 kg (typical adult) and a daily consumption of 1.5 L of non-milk beverages and 750 g of food by a person with a body weight of 15 kg (typical three year-old child) (FAO/WHO, 2009).

- The level of presence of the food enzyme in foods and in non-milk beverages:
The level present in foods and non-milk beverages is assumed to be the highest recommended maximum use level of the food enzyme, expressed in mg of TOS per kg of product (or substrate, or ingredient), reported in any representative category by the applicant, respectively, for foods and beverages. The highest recommended maximum use level is 27.5 mg TOS per kg wheat flour or cereal dry matter for baking and other cereal-based processes and 16.9 mg TOS per kg grits (e.g. barley, malt, wheat, rice) for brewing processes (Table 2).
- The proportion of foods and of non-milk beverages that may contain the food enzyme:
The proportion of solid foods and beverages that may contain the food enzyme is set generally at 25 %. The default proportion (25 %) of beverages and solid food that could contain the enzyme was considered adequate. Although xylanase may be used in a variety of solid foods and beverages that could represent more than 25 % of processed foods, it is unlikely that a person would systematically choose all processed foods with the same enzyme added even considering brand loyalty. This assumes that a typical adult weighing 60 kg consumes daily 1.5 L of beverage and 750 g of solid foods containing the food enzyme.

In case the food enzyme is proposed for products particularly designed for infants or young children, *ad hoc* conservative exposure estimates must be made taking specifically into account these population groups. Otherwise, the very conservative approach described above, is considered to cover both adults and children.

Assuming the conservative scenario described above and also that all processed foods and beverages would contain respectively 70 %¹² and 17 %¹³ of cereal or grain ingredients, the Panel estimated that the theoretical maximum daily intake of the xylanase food enzyme from this genetically modified *A. oryzae* strain would be approximately 0.31 mg TOS/kg bw (27.5 mg TOS/kg x 0.05 kg/kg bw x 25 % x 70 %) + (16.9 mg TOS/kg x 0.1 L/kg¹⁴ bw x 25 % x 17 %).

5. Toxicological data

The toxicological assays were performed with a food enzyme (batch PPJ 6867, see Table 1) representative of the food enzyme before stabilisation and formulation, i.e. before addition of other components such as glycerol and sorbitol.

Genotoxicity testing

In order to investigate the potential to induce gene mutations in bacteria, an Ames test was performed according to OECD Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP) in four strains of *Salmonella typhimurium* (TA1535, TA100, TA1537, TA98) and *Escherichia coli* WP2uvrA, in the presence or absence of metabolic activation by S9 mix applying the “plate incorporation assay”. Two experiments were carried out using six different concentrations of the food enzyme using appropriate positive control chemicals and phosphate buffer as a negative control. The highest concentration was 5 000 µg dry matter/plate, corresponding to ca. 4 100 µg TOS/plate. According to the applicant, all positive control chemicals induced significant increases in revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9 mix, while the negative controls were within the normal ranges. Upon treatment with the food enzyme there was no increase in revertant colony numbers, except for one strain of *Salmonella typhimurium* at the highest concentration of the food enzyme tested. The increased number of revertants was not concentration-related, but was assumed by the applicant to be due to histidine present in the food enzyme. For that reason, the experiment was repeated applying a “treat and plate assay”, in which the *Salmonella typhimurium* strain was exposed to six concentrations of the food enzyme in a liquid medium for 3 hours before plating the centrifuged cells. No increase of revertant colony numbers was observed.

¹² 100 g flour gives 140 g bread.

¹³ In general, 1 kg of grain gives 6 kg of beer.

¹⁴ It is assumed that the densities of beverages are ~1.

Therefore, it was concluded that the food enzyme has no mutagenic activity, under the conditions employed.

The *in vitro* chromosome aberration test carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP. Cultured human peripheral blood lymphocytes, the division of which was stimulated with phytohaemagglutinin (PHA), were treated with purified water (negative control), the food enzyme or appropriate positive controls (4-nitroquinoline 1-oxide or cyclophosphamide in the absence or the presence of the S9 mix, respectively). Two experiments were performed. In the first experiment, applying 3 + 17 hours treatment, the cultures were exposed to the food enzyme (3 200, 4 000 and 5 000 µg food enzyme/ml corresponding to ca. 320, 400 and 500 µg TOS/ml) for 3 hours, either in the presence or the absence of the S9 mix. In the second experiment, the concentrations of the food enzyme tested were 2 813, 3 750 and 5 000 µg food enzyme/ml (corresponding to ca. 280, 380 and 500 µg TOS/ml); a 20 + 0 hours treatment, where cultures were exposed to the food enzyme for 20 hours without the S9 mix and a 3 + 17 hours treatment with the S9 mix, were applied. In all cases, the cells were harvested 20 hours after the beginning of treatment. Only cells with 44 to 46 chromosomes were considered. For all food enzyme concentrations used, the frequency of cells with chromosomal aberrations was similar to that of negative controls (values of $p \leq 0.05$ were considered as significant). It was concluded that the food enzyme did not induce chromosomal aberration in cultured human peripheral blood lymphocytes when tested up to 5 000 µg food enzyme/ml (corresponding to ca. 500 µg TOS/ml) under the experimental conditions employed.

Repeated dose toxicity testing

A 90-day subchronic oral toxicity study was performed according to OECD Test Guideline 408 and following GLP (OECD, 1998). Four groups of 10 male and 10 female SPF Sprague Dawley rats were given by gavage a dose of 10 ml/kg bw/day tap water, a 10 % solution, a 33 % solution as well as an undiluted stock solution of the food enzyme for 13 weeks. The doses were equivalent to 0, 2100, 7700 and 21100 FXU(S)/kg bw/day or 0, 106, 390 and 1 070 mg TOS/kg bw/day.

Blood analysis revealed changes already from the mid dose female's group; statistically significant decrease in red blood cells (RBC) and white blood cells (WBC), as well as a considerable decrease on neutrophils (NEUTRO). A statistically significant increase on the Mean Cell Volume (MCV) in the high dose female's group and a statistically significant decrease on the lymphocytes count in the low and high dose female's group were also observed.

A small increase in the incidence and severity of extramedullary haemopoiesis (EMH) in the spleen was reported in female rats receiving the mid and high dose. A small increase in absolute and relative spleen weight (12 % and 10 %, respectively) which becomes statistically significant in the high dose female's group (23 % and 21 %, respectively) was also reported.

Though the magnitude of some of these effects was small in the mid dose level, the Panel decided that, as they could be considered as likely indicative of the same effects (i.e. decrease in the RBC and effects in spleen) and the system disturbance could increase susceptibility, these changes should be considered adverse. Consequently, the Panel derived a NOAEL on the low dose level of 106 mg TOS/kg bw/day.

6. Allergenicity

Potential allergenicity of xylanase was assessed by comparing its amino acid sequence with those of known allergens according to the scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2010). Using higher than 35 % identity in a window of 80 amino acids as the criterion, no match was found. No food allergic reactions to xylanase have been reported in the literature.

Consequently, the CEF Panel considers that the likelihood of food allergic reactions to this xylanase produced with the genetically modified strain of *A. oryzae* is low and therefore does not raise safety concern.

7. Discussion

The parental strain *A. oryzae* A1560 has a long history of use for the production of food enzymes. The recipient strain, *A. oryzae* BECh2, was developed from the parental strain through a series of modification steps including classical mutagenesis and genetic modification. The *A. oryzae* production strain NZYM-FB contains a recombinant gene encoding xylanase. Multiple copies were randomly integrated into the genomic DNA as part of the plasmid vector. The introduced trait is well-known and does not trigger a safety concern. No sequences that can cause safety concern (e.g. antibiotic resistance genes) remained. Neither the production strain nor the recombinant DNA are detected in the final products by methods considered adequate by the CEF Panel.

The information provided on the manufacturing of the food enzyme, i.e. the fermentation conditions and the steps employed for isolation and purification, is considered sufficient. The available compositional data, including experimental evidence of the absence of potential contaminants such as mycotoxins, sufficiently demonstrate the identity and the purity of the food enzyme. The reported batch-to-batch variability is considered acceptable.

The process conditions provided indicate that the enzyme is inactivated and/or removed in the course of the intended food applications.

Taking the proposed uses and the recommended maximum use levels into account, a dietary exposure assessment based on the Budget Method resulted in a TMDI of 0.31 mg TOS/kg bw. The Panel is aware that the exposure assessment performed is highly conservative because it does not take into account factors such as inactivation and removal of the enzyme during food processing.

The food enzyme produced with the genetically modified *A. oryzae* strain NZYM-FB did not induce gene mutations in bacteria with or without metabolic activation. Neither did it induce chromosome aberrations in cultured human blood lymphocytes.

A comparison of the NOAEL (106 mg TOS/kg bw/day) from the 90-day study with the TMDI (assessment performed according to the Budget method; Section 4), calculated to be 0.31 mg TOS/kg bw/day, results in a margin of exposure (MOE) of 345, which is found sufficient in the light of the conservative interpretation of the effects observed in the 90-day oral toxicity study and the conservative nature of the exposure estimate.

The CEF Panel considers that the likelihood of food allergic reactions to the xylanase from this genetically modified strain of *A. oryzae* is low and therefore does not raise safety concern.

CONCLUSIONS

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided and findings in the toxicological studies, the food enzyme “Xylanase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FB)” does not raise safety concern under the intended conditions of use.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier “Xylanase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FB)”. October 2012. Submitted by Novozymes A/S.
2. Additional information received from Novozymes A/S in June 2013.

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ABBREVIATIONS

Afl	Aflatoxin
AGU	AmyloGlucosidase Units
bw	Body weight
CAS	Chemical Abstracts Service
CFU	Colony Forming Units
CPA	Cyclopiazonic acid
DNA	Deoxyribonucleic acid
EC	European Commission and Enzyme Commission
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Commercial Chemical Substances
EMH	Extramedullary haemopoiesis
EU	European Union
FAO	Food and Agricultural Organisation
FAU	Fungal Alpha-amylase Units
FXU(S)	Fungal Xylanase Units
G	Gram
GLP	Good Laboratory Practice
GMM	Genetically Modified Microorganisms
GMO	Genetically Modified Organisms
GMP	Good Manufacturing Practice
HUT	Hemoglobin Units Tyrosine
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KA	Kojic Acid
kCal	kilo calories
kDa	kilo Dalton
kg	Kilogram
KLU	Kilo Lipase Units
L	Liter
LC-MS/MS	Liquid Chromatography/tandem Mass Spectrometry
MCV	Mean Cell Volume
mg	Milligram
ml	Millilitre
MOE	Margin of exposure
MS	Mass Spectrometry
NOAEL	No Observed Adverse Effect Level
NPA	β -Nitropropionic acid
OECD	Organisation for Economic Cooperation and Development
PAHBAH	<i>p</i> -Hydroxy benzoic acid hydrazide
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
QPS	Qualified Presumption of Safety
RBC	Red Blood Cell
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis
TMDI	Theoretical Maximum Daily Intake
TOS	Total Organic Solids
UV	Ultraviolet
WBC	White Blood Cell
WHO	World Health Organisation